Microarray-based enzyme profiling: Recent advances and applications (Review)[a)]

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Enzymes are an integral part of biological systems. They constitute a significant majority of all proteins expressed (an estimated 18%–29%) within eukaryotic genomes. It thus comes as no major surprise that enzymes have been implicated in many diseases and form the second largest group of drug targets, after receptors. Despite their involvement in a multitude of physiological processes, only a limited number of enzymes have thus far been well-characterized. Consequently, little is understood about the physiological roles, substrate specificity, and downstream targets of the vast majority of these important proteins. In order to facilitate the biological characterization of enzymes, as well as their adoption as drug targets, there is a need for global "-omics" solutions that bridge the gap in understanding these proteins and their interactions. Herein the authors showcase how microarray methods can be adopted to facilitate investigations into enzymes and their properties, in a high-throughput manner. They will focus on several major classes of enzymes, including kinases, phosphatases, and proteases. As a result of research efforts over the last decade, these groups of enzymes have become readily amenable to microarray-based profiling methods. The authors will also describe the specific design considerations that are required to develop the appropriate chemical tools and libraries to characterize each enzyme class. These include peptide substrates, activity-based probes, and chemical compound libraries, which may be rapidly assembled using efficient combinatorial synthesis or "click chemistry" strategies. Taken together, microarrays offer a powerful means to study, profile, and also discover potent small molecules with which to modulate enzyme activity. [DOI: 10.1116/1.3462969]

I. INTRODUCTION

Speed and throughput are key drivers for "-omics" research. Differing from traditional hypothesis driven-research in biology, "-omic" methods are largely discovery-driven and seek to adopt a comprehensive view of the overall system.[1,2] By leveraging on throughput and comparative profiling, this research method generates a wide information base about a particular biological state, within a short space of time.[3] The data generated fuels the next wave of more specific research questions. This adoption of -omic methods has successfully catalyzed discovery and knowledge growth in biology since the 1990s and continues to spur on biomedical and life-science research in the 21st century.[4] Miniaturization and automation further underpin the ability of many -omic platforms to deliver the intended throughput.

The microarray is a platform that delivers phenomenal screening throughput and capabilities.[5–8] The concept at the heart of the technology is elegant, yet simple: by presenting large collections of molecules at a high density on a flat surface, one is able to interrogate them quickly and conveniently, evaluating all possible interactions in a single step. Protein, peptide, and small molecule microarrays have over the last decade been established as a robust tools for screening, lead discovery, and molecular characterization. The identity of each spot is encoded by its position on the array. Anywhere from the hundreds to tens of thousands of samples may be populated on planar surfaces, typically glass or gold-coated slides.[9] The spectrum of applications is determined by the nature and class of molecules immobilized.

DNA microarrays, for instance, were developed in the early-mid-1990s by the groups of Maskos and Brown, and comprised surfaces with addressed oligonucleotides.[10–13] Each spot on the array displays a known DNA sequence. Thousands of them collectively on the microarray act like "probes" to, quantitatively or comparatively, hybridize fluorescently labeled DNA from complex samples. This offers tremendous potential for applications in profiling the expression levels of mRNA and in identifying chromosomal abnormalities, other genetic differences across samples.[14,15] DNA microarrays continue to be widely applied and provide an

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unprecedented view into comparative genomics and genetics.[16] They were the first in a long pipeline of a variety of different microarray types.

As the chemistries improved, a variety of molecules other than DNA, including proteins,[9,17–19] peptides,[6,20,21] carbohydrates,[22–24] and chemical libraries[2,25] were likewise arrayed and presented on microarrays. This happened at the turn of the century, when Schreiber *et al.* developed microarrays containing small molecules in 1999 and proteins in 2000.[26,27] With these exciting developments, it did not take long before proteome arrays,[28,29] cell arrays,[30–32] and tissue arrays[33] also emerged, all within the first quarter of the last decade. The essence of what makes microarray technology so successful is its ability to miniaturize and parallelize assays.[2,34]

Microarrays for enzyme profiling, the topic of this review, describes both synthetic and natural libraries of peptides, carbohydrates, and chemical compound libraries. Simply put, the application of these microarrays is biological screening, but covers specialized applications in protein fingerprinting, ligand discovery, and enzyme-substrate characterization. Being able to assess these interactions in high-throughput thus offers valuable potential for enzyme characterization and drug discovery, discriminates enzymes by their patterns of interactions, and provides insight into molecular interactions and structure-activity relationships.[35,36] Furthermore, if many substrates are arrayed, it provides a window into identifying the most preferred substrate for any given enzyme.[20,37,38] These experiments are maturing to a point that they are not just being carried out with purified targets, but even with enzymes within whole proteomes or cellular lysates,[39–41] in order to interrogate protein-protein interactions and analyze the pathways involved in regulating protein function.[42–45]

Enzymes are an integral part of every cellular process and metabolic exchange. They are proteins that not only sustain life but are also implicit to its regulation and evolution. Minor changes in enzyme activities, either through point mutations or expression changes, are known to cause major diseases (such as cancer, arthritis, Alzheimer disease) and even promote tumor metastasis.[46] Similarly pathogens (including many types of viruses and bacteria) may be targeted by exploiting the uniqueness of their enzymes and metabolic processes.[14,47]

There is hence a pressing need to elucidate the subtle differences that make each enzyme unique. A detailed understanding of the architecture of enzyme active sites facilitates not only the design of potent and selective inhibitors but also the discovery of its biological function and downstream targets.[48] The challenge is in developing cost-efficient and high-throughput means to perform screening and enzyme profiling. Several key components in microarray research and development, mainly library creation, array fabrication, and the instrumentation, have in the past been prohibitively expensive. However, the increased maturity of the platform, the reducing cost and accessibility to compound libraries and the instruments at core facilities, is making it a more cost-effective and viable option for routine screening.[5,49] Microarray fabrication services are also now being provided as a fee-for-service option by commercial vendors, allowing researchers to focus on the downstream application of the technology.[49]

The design of microarray approaches is heavily influenced by the class of enzymes being tested and their associated catalytic function. Kinases, for example, transfer a phosphate group onto a hydroxyl-containing side chain (namely, that of serine, threonine, or tyrosine) within a cognate amino acid sequence of a protein substrate. Phosphatases reverse this process by removing the phosphate group. The interplay between these two groups of enzymes gives rise to the dynamic signal transduction pathways in eukaryotic systems used for inter- and intracellular communications. Proteases on the other hand hydrolyze the peptide bond linking amino acids in proteins. These serve several functions, including protein trafficking and export, as well as in cell-cell signaling and protein degradation. There are over 518 kinases in the human kinome,[50] 1036 proteases in the degradome,[51] and 180 phosphatases in the phosphatome.[52] Before the development of high-throughput tools, it was a significant challenge to study the intricacies among these, as well as the many other classes of enzymes.

This article will describe the design and application of microarray approaches for studying kinases, phosphatases, and proteases, and examine the unique solutions for each class. Section II will include discussions on the chemistries that have made it possible to rapidly synthesize and assemble large assortments of compounds. We will then highlight in Sec. III the technologies that have been developed to accelerate the biochemical screening of various classes of enzymes. Finally we will discuss some of the important biological findings, as well as the future directions of this rapidly maturing field.

II. LIBRARY DESIGN AND FABRICATION

Library design and creation is the first and perhaps the most critical step in any microarray project. For most applications, the libraries involved are either those of the enzymes themselves (which are then expressed and profiled using high-throughput cloning/protein expression and purification strategies) or those of the substrates and/or inhibitors. Substrates and inhibitors are usually fabricated by chemical means and will be elaborated upon in this section. This include libraries of peptides (in the case of kinase and protease substrates), phosphopeptides (in the case of phosphatase substrates), and small molecule peptide mimics (when the intent is to screen enzyme inhibitors). Depending on the enzyme class, specific cognate libraries and sequences are designed, with specific sites along the substrate diversified with natural, or even unnatural, amino acids. The main consideration in designing such a library is balancing its cost against the desired diversity space for enzyme screening and profiling. It almost goes without saying, the larger the library, the greater the cost (in terms of manpower, time and resources required). This has to be weighed against the investment into the library size and diversity, which could lead to a greater likeli-

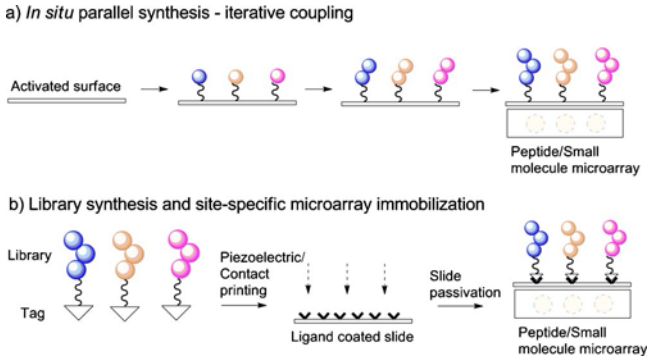

FIG. 1. (Color online) Peptide and small molecule microarray fabrication methods. (a) Iterative coupling for *in situ* library synthesis. (b) Site-specific immobilization of tagged combinatorial libraries, printed on slides using microarray spotters.

hood of meaningful results to discriminate and profile the enzymes of interest. Ideally the chemistries used should be efficient and high yielding, to avoid the need for library purification and yet be at sufficiently high quality for a meaningful first-tier screen.

A. Microarray fabrication approaches

There are in general three fabrication approaches, of relevance to enzyme profiling on microarrays. First, *in situ* creation allows microarrays to be built, usually by iterative couplings of the building blocks, to create the entire library on the slides themselves [Fig. 1(a)]. This is similar to methods developed for SPOT-synthesis, where peptide arrays are built by iteratively coupling amino acid building blocks onto membranes.[53] This was originally demonstrated on glass slides for short peptides through the use of photolithography (with photomasks), a method still in use today for the commercial fabrication of high-density DNA microarrays.[54] To reduce the expenses involved (as photomasks are costly), Gulari *et al.* simplified the process through the use of photogenerated acids that could be activated using precise light-control to facilitate parallel *in-situ* peptide synthesis.[55] This method, however, involved many coupling steps because of the 20 amino acids that have to iteratively coupled on the arrays. A recent development to minimize some of these challenges was developed by Breitling *et al.* using electrically charged solid amino acid particles to efficiently couple the synthesis of all 20 different amino acids in a single step, reducing the cycles needed for array fabrication.[21,56,57] *In situ* methods offer limited opportunities for quality checks or the removal of truncated products; hence the chemistries adopted have to be very efficient to ensure high quality arrays.

Second is the fabrication of libraries, usually with the inclusion of a tag, and the subsequent deposition on the microarray surface using piezoelectric methods or contact printing [Fig. 1(b)]. This is the most common method for microarray fabrication, so it will be discussed at some length here. It is greatly preferred that each library member is known and identified *a priori*, before the arraying process. This, however, imposes a considerable burden to the synthesis phase, as it comes at the expense of added time, effort, and cost. To cater for this, methods have to be built in to ensure that every library member can be identified at the end of the combinatorial synthesis process. Because of the challenges involved, certain groups have sought to use mass spectrometry to deconvolute identified hits only after sample application.[58,59] The adoption of tea-bag style synthesis, using radiofrequency tags, has, however, made it possible to synthesize large chemical libraries, with each member identifiable at the end of the synthesis process.[60]

The synthesis process should also at some point include a tag to enable covalent immobilization on the arrays.[34] If the tag is generic (like a common amine or carboxylic acid or aldehyde) and appears at multiple locations of the molecules, it results in regiospecific immobilization. In this context, any of the tags present within the molecule could bind to the functionalized microarray surface, hence presenting the molecules in one of several possible orientations. This results in a mixed orientation of molecules within a spot. The *p*H of the immobilization buffer used can, for example, favor immobilization of terminal amines in place of epsilon amines of lysines because of their different acid dissociation constants (pKa).

Site-specific immobilization involves a unique tag present at one predefined position in the library. This tag chemoselectively binds the functionalized array surface. For most applications, site-specific immobilization is preferred as the molecules are homogenously presented on the array surface. Where the binding epitopes are unknown, or where one would like to present more facets of the molecules for interaction, regiospecific immobilization may be preferred to not constrain the molecules to any predefined orientation.[61–63] Certain tags may also be applied that facilitate noncovalent, but nevertheless stable immobilization of molecules onto the surface. These include the use of polyhistidine tag (with a nickel or NTA surface), the biotin tag (with an avidin surface), and epitope tag (like flag or glutathione-S-transferase, GST) for immobilization on antibody coated surfaces.[64,65]

The third method of interest for array creation is the use of DNA or PNA-tags for deconvolution [Fig. 3(a)]. These methods adopt the use of DNA microarrays for hit deconvolution and apply peptide nucleic acids or other oligonucleotides that have also been used as tags for small molecule libraries. The positive hits are identified through hybridization onto DNA microarrays. These strategies have been applied to profile the substrates of a variety of serine and cysteine proteases.[66–68] Separately, this has also been creatively applied to study the binding of proteins to multivalent ligands and inhibitors, through directed self-assembly of the probes.[69,70]

B. Chemical synthesis strategies

For most of the methods described herein, combinatorial chemistry has been the key driver catering to the provision of large libraries of compounds. The use of solid supports, split-pool synthesis, and encoding techniques (tea bag synthesis) have facilitated the synthesis of vast libraries of compounds,

including peptides, druglike molecules, and carbohydrates.[71,72] Schreiber *et al.* developed and pioneered various approaches of combinatorial library design and synthesis for microarray fabrication.[26,59] This has included diversity oriented synthesis to generate structurally unbiased libraries as well as one bead-one stock solution split-pool approaches.[73] Synthesis on solid support provides a variety of advantages over solution-phase synthesis. Through automation, repeated coupling cycles can be easily carried out, and the use of such solid supports allows reactions to be driven to completion (by using high concentrations of reactants), ensuring high-yields and purities for standard, well-optimized chemistries. Reactions that give low yields are, however, not amenable to synthesis on solid support, as iterations on solid support will diminish overall yield and purity. Synthetic strategies on solid support include position scanning libraries, alanine-scanning libraries, diversity-oriented synthesis, and other approaches.[74]

Other methods for library fabrication include newer fragment based approaches that enable the assembly of large libraries of diverse molecules (totaling $N^1 \times N^2$) from smaller numbers of building blocks (totaling $N^1 + N^2$), where N is the number of each type of building blocks. This is of particular interest for targeted library synthesis where different inhibitor building blocks may be combinatorially assembled to maximize the diversity space available for screening. "Click chemistry," a term coined by Sharpless *et al.*, is one such fragment-based tool that has received a considerable amount of attention in recent years because of its ease of use and high-efficiency.[75] One of the best known types of "click" reactions is the Cu(I) catalyzed 1,3-dipolar cycloaddition reaction between azides and terminal alkynes.[76,77] Other click reactions include the Staudinger ligation which has also been applied in array fabrication.[78,79]

We have found it very convenient to apply highly strategies such as "click chemistry" and amide-forming reactions.[80] Here the different building blocks of enzyme substrates, inhibitors or activity-based probes, may be conveniently assembled together at the final stages of the synthetic process, in contrast to bottom up synthesis. Such a modular design and synthesis greatly expands the catalomic toolbox by enhancing the speed and efficiency of library fabrication. Similar library sets may be designed with different handles for activity-based probes in one study and also be used to generate combinatorial libraries of substrates or inhibitors in another, hence avoiding duplication of the synthetic effort. Click reactions are mild and highly efficient, often providing quantitative yields. This facilitates "one-pot" style synthesis and screening, where the reaction products may be synthesized and used directly *in situ*, for biochemical and microarray-based screening.

In the early years, the investment in expensive equipment and reactors for combinatorial synthesis was one barrier limiting access to microarray technology. Over the last decade, improved chemistries for synthesis and immobilization, coupled with commercial support, now brings about greater access to robust library and microarray fabrication. Prefabricated arrays and coated microarrays slides, and even microarray vendors who now offer complete design and array fabrication packages, will make the platform more accessible to interested users.[49]

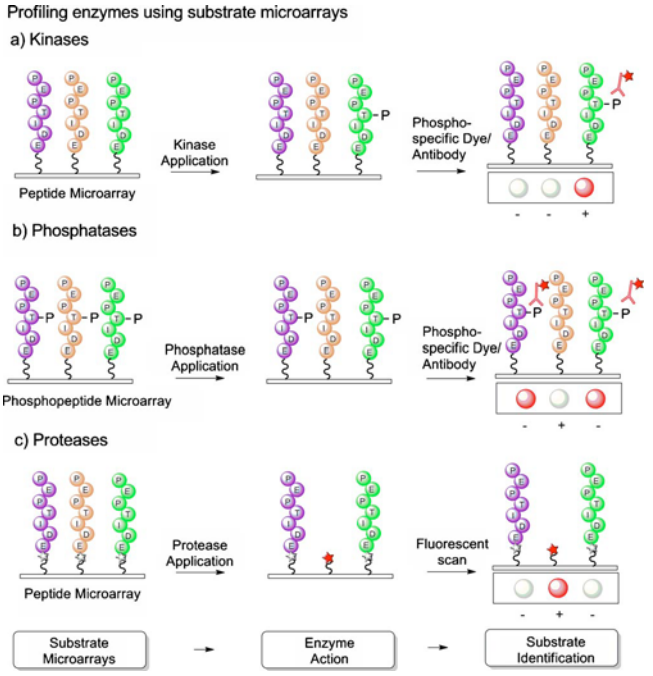

FIG. 2. (Color online) Profiling enzymes using substrate microarrays. (a) Fluorogenic peptide microarrays for protease profiling. (b) Kinases are screened against peptide substrate microarrays that contain the relevant amino acid residue (e.g., Ser, Thr, or Tyr). (c) Phosphatases may be screened against phosphopeptide arrays. In this case, the reduction of signal is indicative of the preferred substrate.

III. MICROARRAY-BASED SCREENING AND APPLICATION

The landmark papers by Schreiber and Snyder in 2000 and 2001 established that proteins can retain their activity even when physically immobilized on microarrays.[27,29] This broke a long standing paradigm that macromolecular proteins with complicated and delicate structures would be denatured when immobilized on solid surfaces. Many groups have since shown that enzymes, even covalently immobilized on such array substrates, still retain their activities and can be thus studied in parallel. Of course, a great deal of care needs to be taken when working with enzymes under these conditions. Certain enzymes may also be very delicate and could quickly lose activity once immobilized on arrays. The use of linkers and three-dimensional arrays (hydrogels) have also aided in preserving the activity of proteins on microarrays.[81,82]

A. Substrate profiling

Early work by Ellman *et al.* showcased ways in which peptide microarrays may be applied to profile proteases [Fig. 2(a)].[83] By taking advantage of fluorogenic peptide substrates anchored on microarrays, the study was able to test

for the activities of several proteases, including trypsin, granzyme B, and thrombin. The coumarin-based derivatives facilitated the profiling of nonprime (*N*-terminal segment) of the substrate recognition motif to be explored, through monitoring the cleavage of the anilide bond that liberated strong coumarin fluorescence. Our group also reported a broader application of coumarin derivatives on microarrays to study proteases as well as esterases, phosphatases, and other hydrolases on microarrays.[84] Besides hydrolases, Park and Shin developed microarray methods to assess the activities of glycosyltransferases.[85] Oh *et al.* demonstrated the analysis of sumoylation (SUMO-small molecule ubiquitin modifier) using microarrays.[86]

Diamond *et al.* were the first to develop a droplet-based method using microarrays to assay the substrate specificity of serine and cysteine proteases in which fluorogenic substrates and enzymes are sprayed in aerosol form onto the slides.[37,38] A 722-member coumarin peptide library was deposited in glycerol droplets on the microarrays, thereafter the proteases were delivered to the arrays in an aerosolized spray. The platform was applied to characterize the substrate spectrum of rhodesein, a papainlike cysteine protease from the parasite, *Trypanosomes brucei*.[37] It was also used to distinguish the substrate preferences of thrombin obtained from various sources. A 361-member subset of the peptide library was also applied to characterize proteolytic activity of human plasma.[87] Alternative surfaces have further been developed for droplet-based enzyme profiling.[88–90] Using slides coated with fluorogenic substrates, activity-dependent profiles of proteases and phosphates may obtained in nanoliter sized droplets, in a time- and concentration-dependent manner.[90] This method was applied to screen for inhibitors of metalloproteases, namely, thermolysin and collagenase, using nearly 150-fold less substrate in comparison to the traditional microplate method.[20] A similar droplet-based method has been developed using sequential piezoelectric deposition with larger droplet sizes (100–150 nl).[91]

Apart from hydrolases, methods have also been developed to screen the substrate specificity of kinases using peptide microarrays [Fig. 2(b)]. The use of fluorescently labeled antiphosphoserine and antiphosphotyrosine antibodies, or phosphospecific dyes has overcome the traditional need for radioactive methods to detect kinase activity.[92–94] We studied the use of combinatorial peptide libraries to obtain the activities of a kinase p60-Src, demonstrating the possibility of using such peptide libraries to screen for kinase substrate specificity.[95] This was significant as it demonstrated that apart from conventional one-spot one-compound style approaches, one-spot many-compound approaches could also be used for informative screening on peptide microarrays. An alternative was the use of a phosphospecific dye, Pro-Q Diamond, for the highly sensitive measurement of peptide phosphorylation on microarrays.[96]

Various groups have pursued large scale kinase screening initiatives, not only to understand the target spectrum of kinases but also to uncover their underlying roles in signal transduction pathways under diverse physiological states. By leveraging on methods by which the phosphorylated peptide motifs could be distinguished from the unphosphorylated peptides, kinases could readily be profiled using peptide microarrays. Expanding on the scale and content, Schutkowski *et al.* built a large scale microarray for kinase screening which included 6912 peptides on a single chip.[97,98] An *N*-terminal aminoacetyl tag was used to facilitate covalent attachment to aldehyde slides. The library comprised 710 human phosphorylation sites incorporated as 13-mer peptide sequences. The microarrays were screened against protein kinase A (PKA), casein kinase 2 (CK2), and 3-phosphoinositide-dependent protein kinase (PDK1) to successfully reveal preferred target sequences/motifs. The same group also reported the use of 13 000 peptides across six microarray slides to profile casein kinase 2 (CK2) and Abl kinase.[99,100]

Wang *et al.* studied the transmembrane KPI-2 kinase, a member of the human lemur kinase family, using a 1154 member peptide microarray.[101] They uncovered that despite its putative classification as a Tyr kinase (by sequence homology), KPI-2 specifically phosphorylated only Ser/Thr containing peptides on the microarray. The best substrate corresponded to Ser-737 in the regulatory domain of the cystic fibrosis transmembrane conductance regulator. Brock *et al.* adopted a unique approach for exploring signal transduction on microarrays. By applying cell lysates under differential states, they developed a model system to investigate T-cell signaling.[102] In another application that utilized cellular lysates, Katayama *et al.* demonstrated the ability to monitor the activities of both PKA and PKC simultaneously.[40]

Phosphopeptide libraries have also been fabricated and immobilized on microarrays to test the activities of phosphatases, in assaying their ability to remove the phosphate group from specific amino acid sequences [Fig. 2(c)].[103,104] These strategies have enabled the screening of serine and threonine phosphatases (PP2A, Lambda, PP1) using microarrays. A phosphospecific dye (Pro-Q Diamond) was used to detect the signal decrease from the phosphopeptide library as a result of dephosphorylation by phosphatases. Distinct phosphatase profiles were obtained using a panel of 89 different phosphopeptides, which were immobilized using a biotin tag onto streptavidin coated slides. Unique profiles were further obtained upon coincubation of a phosphatase, PP2A with a potentially regulatory peptidyl-prolyl isomerase Pin1, demonstrating that this strategy is applicable in studying the regulatory role of different proteins within the signal transduction cascade. This led to the discovery that the dephosphorylation of a protein involved in apoptosis (Bcl-2) is regulated by both Pin1 and PP2A.[103] Waldmann *et al.* applied Staudinger ligation to chemoselectively ligate 48 phosphorylated peptides onto phosphane glass surfaces. The arrays were screened with protein tyrosine phosphatases (PTP1B and PTPμ) and probed with a fluorescently tagged antiphosphotyrosine antibody, to assess signal reduction as a result of phosphatase activity.[104]

We recently developed a "substrate trapping" peptide microarray which facilitated the substrate screening of putative

protein tyrosine phosphatases (PTPs), and determination of the potent and specific binders of different PTPs.[105] This was achieved by replacing the conserved active site cysteine residue with serine, to generate a substrate-trapping mutant that retains the substrate recognition property but loses its dephosphorylation ability. As a result, the enzyme is linked with its substrate of choice. Using dual-channel dye labels, we intercompared the substrate binding profiles of phosphatase mutants and were able to discern very subtle differences in the substrate binding of two closely related phosphatases (i.e., PTP1B and TCPTP). A putative peptide sequence derived from mitogen kinase was found to preferentially bind PTP1B over TCPTP. This may have potential therapeutic implications.

B. Inhibitor profiling and protein fingerprinting

Mihara *et al.* were among the earliest to develop protein fingerprinting approaches using immobilized peptides on microarrays.[106,107] A 112-member α-helical peptide microarray was used to distinguish seven proteins. In another example, Kodadek and Reddy developed high-density microarrays comprising 7680 octameric peptoids, which were applied in the large-scale protein fingerprinting of three model proteins.[108] In this case, the library was designed in a manner in which the identities of the library members remain unidentified, preventing functional binding data to be elucidated from the protein binding profiles. Microarrays have similarly been applied for inhibitor identification, particularly for caspases,[109] cysteine proteases,[110] and metalloproteases.[20]

Through the use of activity-based-probes, protein arrays may be screened and tested for enzyme activity and inhibition [Fig. 3(b)].[111] We first demonstrated the method using a series of enzymes immobilized on epoxy slides, including serine proteases, cysteine proteases, and phosphatases.[112] These enzymes were screened against activity-based-probes, which covalently bound only active proteins. The probes carried a fluorescent reporter tag, to enable visualization of the active enzyme spots within the microarray. Schmidinger *et al.* demonstrated a similar strategy against lipolytic enzymes.[113] Taking the strategy further, Miyake *et al.* were able to study quantitative inhibitor kinetics by adding various concentrations of inhibitors to the probe mixture and monitoring the probe labeling efficacy in a time-dependent manner on the microarrays. This enabled the on-chip determination of inhibition constants.[114,115]

Koehler *et al.* developed a library of compounds that resembled suberoylaniline hydroxamic acid (SAHA).[116] This is a compound that inhibits members of the histone deacetylase (HDAC) family of enzymes. Conjugated using fluorous tags, a library of 20 SAHA-like compounds were immobilized on fluorinated slides. HDACs catalyze the hydrolysis of *N*-acetyl groups on lysine residues found in the *N*-terminal tails of histone proteins. Binding profiles generated using the small molecule microarrays enabled the identification of selective inhibitors against different HDAC homologs. It was also demonstrated that HDACs present in cell lysates could be profiled and detected on the microarrays using fluorescently labeled antibodies.

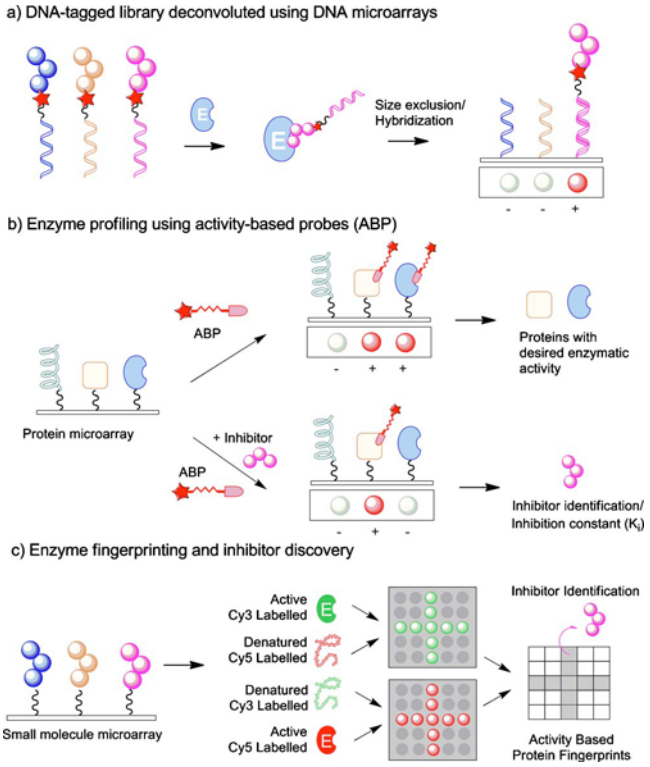

FIG. 3. (Color online) Microarray-based enzyme fingerprinting and inhibitor discovery. (a) The application of PNA/DNA tagged libraries and DNA microarrays for hit deconvolution. (b) Use of activity-based probes for enzyme annotation and inhibition profiling. (c) Inhibitor discovery and enzyme fingerprinting using small molecule microarrays.

Building on these approaches, we found that the false positive rate on microarrays could be significantly alleviated by applying a two-color protein application (where the same protein is labeled in two channels, one channel is left untreated while the other is heat denatured; both channels are applied simultaneously on the arrays). Spots in the denatured channel that came up bright usually indicated false positive interactions that we reasoned were most likely not associated with the functional activity of the protein [Fig. 3(c)]. Subtracting these signals away from the active channel provides a data set that is more representative of the actual functional profile of enzymes. We applied this approach to comparatively profile four metalloproteases (namely, thermolysin, carboxypeptidase A, collagenase, and anthrax lethal factor). The microarrays comprised 1400 hydroxamate peptides (hydroxamate was chosen as the zinc binding group, to target the enzyme active site) that were permuted across the prime positions and site-specifically immobilized on avidin-coated surfaces.[117] Concentration dependent protein application further enabled K_D data to be obtained for hundreds of inhibitors simultaneously on the microarrays. The identity of each of the inhibitors was known *a priori* (unlike the earlier synthetic approach by Kodadek *et al.*[108]). This enabled preferred peptide ligands to be identified for each of the proteins fin-

gerprinted, directly from the high-throughput screening results.

Taking the two-color strategy one step further, we intercompared closely related proteins using microarrays. Across slide comparisons can be a great challenge because of slide-to-slide variations; hence comparing two proteins on the same slide allows actual functional differences to be more readily elucidated. A 1000-member fragment-based phosphoserine/threonine heptapeptide library was generated to screen the activity of the family of seven human 14-3-3 proteins on microarrays, which are important cellular regulators that bind to phosphoserine-containing proteins.[118] 14-3-3 proteins are very closely related, and it has been a challenge to identify their unique target specificities. The microarray-based two-color screening method revealed a novel target sequence that bound 14-3-3 Sigma, which is a protein implicated in tumorigenesis.[118]

In another interesting development, Dordick *et al.* developed cytochrome P450 (CYP) arrays that are able to screen for enzyme activity and inhibition. In one demonstration, sol-gel encapsulated P450 enzymes on microarrays were used to activate a prodrug, cyclophosphamide. These microarrays were then stamped with a MCF7 cell monolayer (a breast cancer cell line) against which the drugs showed cytotoxicity, if these molecules were appropriately converted by the relevant CYP isoforms.[119] More recently, coupled with high-throughput array imagers, the group has developed fluorescent assays to study the activity of encapsulated CYP enzymes on microarrays.[120]

IV. CONCLUSION

In conclusion, we have herein showcased how microarray methods can be adopted to facilitate investigations into enzymes and their properties, in a high-throughput manner. Using examples of different enzymes, particularly kinases, phosphatases, and proteases, we have described experimental designs and microarray construction to characterize each enzyme class. As a result of research efforts over the last decade, these groups of enzymes have become readily amenable to microarray-based profiling methods. It has become routinely possible to implement high-throughput screening assays for these as well as many other classes of enzymes. This is already contributing to the large-scale assimilation and integration of data, to provide for a future where simulation and prediction of biological effects of molecular interaction *in silico* may be completely possible. With the great progress made over the last decade, the field of microarray-based enzyme profiling continues to expand at a remarkable pace.

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